MANUAL

Technology: AlphaLISA™

AlphaLISA Human PICP Detection Kit

Part number:	AL3208HV	AL3208C	AL3208F
Assay points:	100	500	5,000

Storage: Store kit in the dark at 4 °C. For reconstituted analyte, aliquot and store at -80 °C. Avoid freeze-thaw cycles.

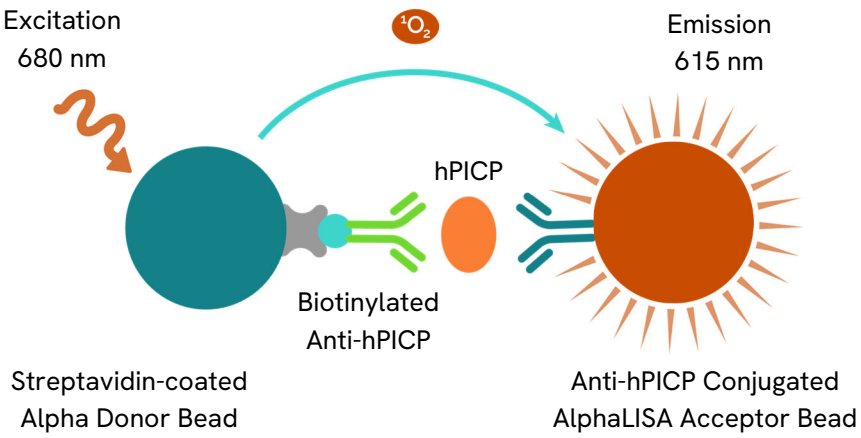
Version: 1 Date: April 2025

ANALYTE OF INTEREST

Collagen Type 1 is a protein present in abundance in the extracellular matrix, where forms cross-linking fibrils that associate in larger collagen fibers to play a role of structural support in connective tissues such as cartilage, bone, tendon, and skin. Due to its very usoluble nature in its mature form, collagen is expressed and transported as a pro-collagen, in which the mature collagen sequence is flanked by two propetides respectively designated as PINP (Pro-collagen type 1 N-terminal Propeptide) and PICP (Pro-collagen type 1 C-terminal Propeptide). Mature collagen detection and quantification is not possible in immunoassays due to it being unsoluble and sticky. Instead, detection of the whole pro-collagen form or one of the propeptides is a good proxy to quantify the collagen expression in cells. In particular, targeting a propeptide like PICP allows to quantify both pro-collagen that have been expressed but not yet processed into mature collagen, and free PICP which have been cleaved from the mature sequence and are markers of collagen deposited in the extracellular matrix. The present kit is designed for the quantitative detection of Human PICP (Pro-Collagen 1 C-Terminal Pro-Peptide) in supernatants.

DESCRIPTION OF THE ALPHALISA ASSAY

AlphaLISA technology allows the detection of molecules of interest in cell culture supernatant in a highly sensitive, quantitative, reproducible, and user-friendly mode. In this AlphaLISA assay, a biotinylated anti-human PICP antibody (anti-hPICP) binds to the streptavidin coated AlphaLISA Donor beads, while an anti-human PICP antibody is conjugated to AlphaLISA Acceptor beads. In the presence of human PICP, the beads come into proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer within the Acceptor beads, resulting in emission with λ_{max} at 615 nm.



PRECAUTIONS

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters) can be applied to light fixtures.
- Take precautionary measures to avoid contamination of the reagent solutions.

- The biotinylated anti-hPICP antibody contains sodium azide. Contact with skin or inhalation should be avoided.

KIT CONTENT: REAGENTS AND MATERIALS

Kit components	AL3208HV***	AL3208C****	AL3208F****
AlphaLISA Anti-hPICP Acceptor beads stored in PBS, 0.05% Kathon CG/ICP II, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	50 µL @ 5 mg/mL (1 brown tube, white cap)	500 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon CG/ICP II, pH 7.4	50 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)	2 x 1 mL @ 5 mg/mL (2 brown tubes, black caps)
Biotinylated Anti-hPICP Antibody stored in PBS, 0.1% Tween-20, 0.05% NaN_3, pH 7.4	20 µL @ 500 nM (1 tube, black cap)	50 µL @ 500 nM (1 tube, black cap)	500 µL @ 500 nM (1 tube, black cap)
Lyophilized Recombinant Human PICP	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)
AlphaLISA Immunoassay Buffer (10X)	2 mL, 1 small bottle	10 mL, 1 medium bottle	100 mL, 1 large bottle

* Reconstitute lyophilized analyte in 100 µL Milli-Q® grade H_2O. IMPORTANT: do not vortex the analyte. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped 0.5 mL polypropylene vials and stored at -80 °C for future experiments. Refer to the product CoA for stability information on the reconstituted analyte stored at -80°C Avoid freeze-thaw cycles. One vial contains an amount of analyte sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # AL3208S).

** Extra buffer can be ordered separately (cat # AL3208C: 10 mL, cat # AL3208F: 100 mL).

*** The number of assay points is based on an assay volume of 100 µL in 96-well plates.

**** The number of assay points is based on an assay volume of 50 µL in 384-well assay plates using the kit components at the recommended concentrations.

Sodium azide should not be added to the stock reagents. High concentrations of sodium azide (> 0.001% final in the assay) might decrease the AlphaLISA signal. Note that sodium azide from the biotinylated anti-hPICP antibody stock solution will not interfere with the AlphaLISA signal (0.0001% final in the assay).

Additional reagents and materials:

The following materials are recommended but not provided in the kit:

Item	Suggested source
Light gray AlphaPlate™-384	Revvity Inc.
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.
EnVision®-Alpha Reader	Revvity Inc.

RECOMMENDATONS

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000*g*, 10-15 sec).
- Re-suspend the Donor and Acceptor beads by vortexing before use.
- Use Milli-Q® grade H_2O to dilute buffers and to reconstitute the lyophilized analyte. Do not vortex the analyte.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Film to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film in place.
- The AlphaLISA signal is detected with an EnVision Multimode Plate Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate. It is recommended to avoid multiple reads of the same well of the assay plate.
- The standard curves shown in this manual are provided for information only. A standard curve must be generated for each experiment.

ASSAY PROCEDURE

- The protocol described below is an example for generating one standard curve in a 50 µL final assay volume (48 wells, triplicate determinations). The protocols also include testing samples in 452 wells. If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly, as shown in the table below. These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes.

- The standard dilution protocol is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

Format	# Of data points	Volumes for Kit Protocol				Plate recommendation*
		Final	Sample	AlphaLISA Acceptor Beads + biotinylated Ab Mix	SA-Donor beads	
AL3208HV	100	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96 White ½ AreaPlate-96
AL3208C	250	100 µL	10 µL	40 µL	50 µL	White OptiPlate-96
	500	50 µL	5 µL	20 µL	25 µL	½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate™-384
	1 250	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate™-384 Plus White OptiPlate-384
	2 500	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536
AL3208F	5 000	50 µL	5 µL	20 µL	25 µL	½ Area AlphaPlate-96 White OptiPlate-384 Light gray AlphaPlate-384
	12 500	20 µL	2 µL	8 µL	10 µL	Light gray AlphaPlate-384 ProxiPlate-384 Plus White OptiPlate-384
	25 000	10 µL	1 µL	4 µL	5 µL	Light gray AlphaPlate-1536

*Light gray AlphaPlates were specifically designed for use with AlphaLISA assays and are strongly recommended for best assay performance.

The protocol described below is for 500 assay points including one standard curve (48 wells) and samples (452 wells). *If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly.*

1) Preparation of 1X AlphaLISA ImmunoAssay Buffer:
Add 1 mL of 10X AlphaLISA ImmunoAssay Buffer to 9 mL Milli-Q® grade H_2O.

2) Preparation of Human PICP analyte standard dilutions:
   a. Reconstitute lyophilized [Human PICP, 0.1µg] in 100 µL Milli-Q® grade H_2O. Important- Do not vortex the analyte. The remaining reconstituted analyte should be aliquoted immediately and stored at -80 °C for future assays (see page 2 for more details).
   b. A standard curve must be generated for each experiment. The standard curve should be performed in a similar matrix diluent as the samples (e.g. cell culture media for cell

supernatant samples). Use of the 1X AlphaLISA ImmunoAssay Buffer is recommended as a diluent to confirm assay performance.

c. Prepare standard dilutions as follows (change tip between each standard dilution).

Tube	Vol. of Human PICP (µL)	Vol. of diluent (µL)*	[Human PICP] in standard curve	
			(g/mL in 5 µL)	(pg/mL in 5 µL)
A	10 µL of reconstituted Human PICP	90	1.00E-07	100 000
B	60 µL of tube A	140	3.00E-08	30 000
C	60 µL of tube B	120	1.00E-08	10 000
D	60 µL of tube C	140	3.00E-9	3000
E	60 µL of tube D	120	1.00E-9	1000
F	60 µL of tube E	140	3.00E-10	300
G	60 µL of tube F	120	1.00E-10	100
H	60 µL of tube G	140	3.00E-11	30
I	60 µL of tube H	120	1.00E-11	10
J	60 µL of tube I	140	3.00E-12	3
K	60 µL of tube J	120	1.00E-12	1
L	60 µL of tube K	140	3.00E-13	0.3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* Dilute standards in appropriate diluent (e.g. 1X AlphaLISA ImmunoAssay Buffer).

At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL/LLOQ is calculated. If LDL/LLOQ does not need to be calculated, one background point in triplicate can be used (3 wells).

3) <u>Preparation of 2.5X MIX AlphaLISA Anti-hPICP Acceptor beads (25 µg/mL) + Biotinylated Anti-hPICP Antibody (2.5 nM):</u>

a. Prepare just before use.

b. Add 10 µL of 5 mg/mL AlphaLISA Anti-hPICP Acceptor Beads and 10 µL of 500 nM Biotinylated Anti-hPICP Antibody to 1980 µL of 1X AlphaLISA ImmunoAssay Buffer.

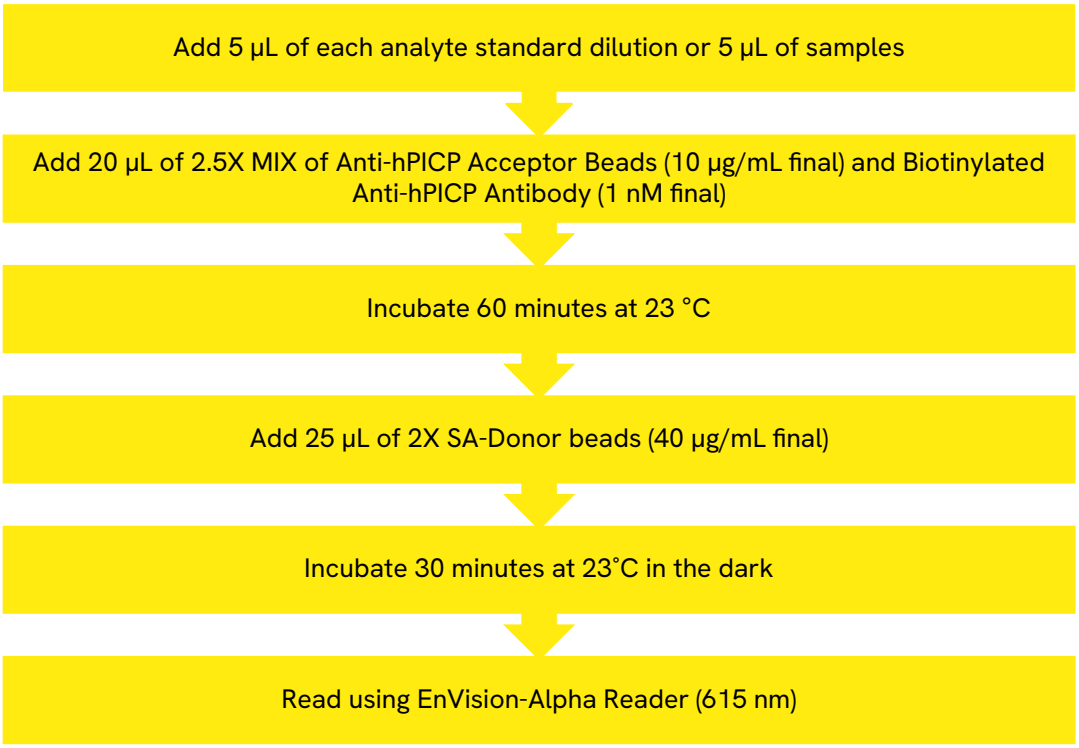
4) Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):

a. Prepare just before use.

b. Keep the beads under subdued laboratory lighting.

c. Add 40 µL of 5 mg/mL SA-Donor beads to 2460 µL of 1X AlphaLISA ImmunoAssay Buffer

5) In a light gray AlphaPlate (384 wells):



DATA ANALYSIS

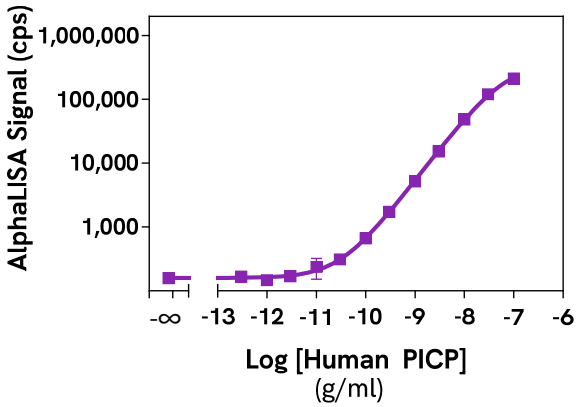
- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + [3xSD]) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + [10xSD]) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

ASSAY PERFORMANCE CHARACTERISTICS

AlphaLISA assay performance described below was determined by using the recommended kit protocol using 1X AlphaLISA ImmunoAssay Buffer as an assay buffer. The analytes (standards) were prepared in different matrix diluents depending on sample type. All other components were prepared in 1X AlphaLISA ImmunoAssay Buffer.

Standard curve:

A typical sensitivity curve is shown below, using the recommended kit protocol described on page 4 to 6, using 1X AlphaLISA ImmunoAssay Buffer to dilute the standard.



Assay sensitivity:

The LDL was calculated by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value (average background counts + [3xSD]) on the standard curve. The values correspond to the lowest concentration of analyte that can be detected in a volume of 5 µL of sample using the recommended assay conditions.

The LLOQ was calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + [10xSD]) on the standard curve. The values correspond to the lowest concentration of analyte that can be accurately quantified in a volume of 5 µL of sample using the recommended assay conditions. Media must NOT be supplemented with FBS as it contains free PICP.

Analyte diluent	LDL (pg/mL)	LLOQ (pg/mL)
AlphaLISA ImmunoAssay buffer 1X	21	66
MEM	81	239
DMEM	64	168
RPMI	126	452

Assay precision:

Samples containing different concentrations of analyte were prepared in MEM without FBS. All other components were prepared in AlphaLISA ImmunoAssay Buffer. The assays were performed in a 384-well plate format.

Intra-assay precision:

The intra-assay precision was determined using a total of 24 replicates per sample in one assay. Shown as CV% of measured concentration.

Human PICP CONCENTRATION	MEM
Sample A, 31 000 pg/mL	9.8%
Sample B, 12 800 pg/mL	5.3%
Sample C, 3 600 pg/mL	4.6%

Inter-assay precision:

The inter-assay precision was determined using a total of 3 independent determinations with three measurements per sample in each assay. Shown as CV% as measured concentration.

Human PICP CONCENTRATION	MEM
Sample A, 43 240 pg/mL	5.1%
Sample B, 14 090 pg/mL	7.1%
Sample C, 4 690 pg/mL	5.0%

Spike and recovery:

Known concentrations of standard (exogenous analyte) were spiked into cell supernatants containing the endogenous analyte. Non-spiked and spiked samples were measured in the assay. Note that the respective standard curves were prepared by diluting the analyte in the appropriate matrix (MEM without FBS). All other assay components were diluted in AlphaLISA ImmunoAssay Buffer.

Good recoveries were achieved for all spiked samples. The results are shown in the table below.

Sample type	[Endogenous Human PICP] in sample (pg/mL)	[Exogenous Human PICP] spiked into sample (pg/mL)	Expected [Human PICP] in spiked sample (pg/mL)	Measured [Human PICP] in spiked sample (pg/mL)	Recovery (%)
Sample 1 (e.g. BJ cell supernatant)	20 796	166	20 962	19 054	91
	20 796	1442	22 238	17 947	81
	20 796	25 822	46 618	39 994	86

Dilution linearity:

A sample (e.g. cell supernatant) collected from BJ cell culture (e.g. fibroblasts derived from neonatal male foreskin) and containing a known concentration of analyte was serially diluted in MEM without FBS. The assay was performed on neat and serially diluted samples, along with a standard curve prepared in the same matrix. Concentrations of Human PICP in samples were determined by interpolating from the standard curve. The other components of the assays (anti-hPICP Acceptor beads, biotinylated anti-hPICP antibody, and SA-Donor beads) were prepared in 1X AlphaLISA ImmunoAssay Buffer.

Excellent dilution linearity ($R^2 > 0,99$, slope = 1.0) and dilution recovery were achieved in the diluted samples (in the range of 2- down to 6-fold dilution). The results are shown in the table below.

Sample 1 (e.g. BJ cell supernatant	Sample dilution factor	Expected [Human PICP] (pg/mL)	Measured [Human PICP] (pg/mL)	Dilution Recovery (%)
	neat	-	35 411	-
	1/2	17 706	20 278	115
	1/4	8 853	10 421	118
	1/8	4 426	4 865	110
	1/16	2 213	2 467	111
	1/32	1 107	1 287	116
	1/64	553	636	115

Human serum/plasma/CSF experiments:

No experiment can be run in FBS or plasma as it contains free PICP and will therefore bias the assay.

TROUBLESHOOTING

To find detailed recommendations for common situations you might encounter with your AlphaLISA assay kit, please visit our website at www.revvity.com.

www.revvity.com

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